

# High Resolution Crystal Structure of Human Rab9 GTPase

A NOVEL ANTIVIRAL DRUG TARGET\*

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Rab GTPases and their effectors facilitate vesicular transport by tethering donor vesicles to their respective target membranes. Rab9 mediates late endosome to trans-Golgi transport and has recently been found to be a key cellular component for human immunodeficiency virus-1, Ebola, Marburg, and measles virus replication, suggesting that it may be a novel target in the development of broad spectrum antiviral drugs. As part of our structure-based drug design program, we have determined the crystal structure of a C-terminally truncated human Rab9 (residues 1–177) to 1.25-Å resolution. The overall structure shows a characteristic nucleotide binding fold consisting of a six-stranded  $\beta$ -sheet surrounded by five  $\alpha$ -helices with a tightly bound GDP molecule in the active site. Structure-based sequence alignment of Rab9 with other Rab proteins reveals that its active site consists of residues highly conserved in the Rab GTPase family, implying a common catalytic mechanism. However, Rab9 contains seven regions that are significantly different in conformation from other Rab proteins. Some of those regions coincide with putative effector-binding sites and switch I and switch II regions identified by structure/sequence alignments. The Rab9 structure at near atomic resolution provides an excellent model for structure-based antiviral drug design.

Rab proteins, the largest subfamily of the Ras-like small GTPase superfamily, serve as molecular switches mediating tethering, docking, fusion, and motility of intracellular membranes (1). Rabs cycle between GTP-bound (on/active) and GDP-bound (off/inactive) conformations (2–4). The active form is stabilized by additional hydrogen bond interactions with the  $\gamma$ -phosphate of GTP mediated by serine residues in the phosphate-binding loop and switch I region as well as an extensive hydrophobic interface between the switch I and II regions (5, 6). The inactive conformation usually has displaced and mobile switch regions (3, 7). Biochemical and genetic studies of chimeric and mutant Rab proteins have identified several hyper-

variable regions, including the N and C termini and the  $\alpha 3/\beta 5$  loop, that play an important role in determining functional specificity (8, 9).

The Rab9 GTPase is localized predominantly to late endosomes and is required for the transport of mannose 6-phosphate receptors from endosomes to the trans-Golgi network (10, 11). Rab9 facilitates vesicular transport by pairing with its cognate Rab effector P40 (12). Rab9 also interacts with the vesicle cargo selection protein TIP47, which has been shown to bind the cytoplasmic tail of the HIV<sup>1</sup> envelope glycoprotein subunit gp41 (13). By targeting Rab9 mRNA for degradation with small interfering RNA, Rab9 has just been identified to be a key cellular component for HIV-1, Ebola, Marburg, and measles virus replication,<sup>2</sup> suggesting that inhibitors of Rab9 function, if developed, might prove useful in the control of those viruses. As part of a new structure-based antiviral drug design program, we have determined the crystal structure of a C-terminally truncated human Rab9 (residues 1–177) to near atomic resolution of 1.25 Å.

## EXPERIMENTAL PROCEDURES

**Cloning and Expression**—The gene for human Rab9 (GenBank<sup>TM</sup> accession number NM\_004251) was obtained from the IMAGE clone collection (IMAGE ID number 4139714) through distribution by Open-Biosystems. A C-terminally truncated fragment coding for residues 1–177 (20.1 kDa) was PCR subcloned using primers 5'-G ACA GCT AGC ATG GCA GGC AAA TCA TCA CTT TTT AAA G-3' and 5'-C ATG GAT CCT TCA GTC CTC GGT AGC AAG AAC TCT TC-3' into the NheI/BamHI restriction sites of pET28b (Novagen); the resulting construct encodes for a Rab9(1–177) protein product with an N-terminal His<sub>6</sub>-containing fusion (MGSSHHHHHHSSGLVPRGSHMAS). The pET28-Rab9(1–177) vector was transformed into *Escherichia coli* BL21(DE3) (Novagen), and overproduction of the fusion protein was induced at an  $A_{600\text{ nm}}$  of  $\sim 2.0$  with 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside at 310 K for 2 h; the cells were harvested by centrifugation and frozen at 253 K.

**Protein Purification**—The cell pellet was resuspended in nickel buffer A (20 mM Tris, pH 8.0, 500 mM NaCl, 5 mM imidazole), lysed by sonication, and centrifuged at  $20,000 \times g$  for 20 min at 277 K. The soluble fraction was filtered through a 0.45-micron filter and applied to chelating Sepharose (Amersham Biosciences), which had been previously charged with 50 mM NiSO<sub>4</sub> and equilibrated with nickel buffer A. The column was then washed with nickel wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 55 mM imidazole), and the His<sub>6</sub>-Rab9(1–177) fusion protein was eluted with nickel elution buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 350 mM imidazole). The His<sub>6</sub>-Rab9(1–177) fusion protein was then dialyzed against Thr buffer (20 mM Tris-HCl, pH 8.4,

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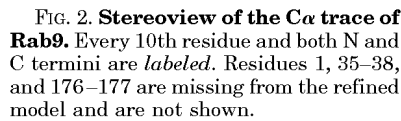
The atomic coordinates and structure factors (code 1WMS) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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<sup>1</sup> The abbreviations used are: HIV, human immunodeficiency virus; MES, 4-morpholineethanesulfonic acid; r.m.s., root mean square; Gpp(NH)p, guanosine-5'-( $\beta$ , $\gamma$ )-imidotriphosphate; Gppsp, 5'-guanosine-diphosphate-monothiophosphate.

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FIG. 1. **Amino acid sequence of human Rab9 and assignment of its secondary-structure elements.**  $\alpha$ -Helix and  $\beta$ -sheet regions defined by the crystal structure are *underlined* and *labeled* ( $\alpha$ -helices starting with *H* and  $\beta$ -strands with *B*).



Data and phasing statistics	
Space group	P <sub>1</sub>
Unit cell: <i>a</i> , <i>b</i> , <i>c</i> (Å), $\alpha$ , $\beta$ , $\gamma$ (°)	38.40, 45.62, 51.22, 99.7, 107.2, 101.8
Wavelength (Å)	1.00
Resolution (Å)	1.25
Reflections (total/unique)	507,012/73,993
Completeness (%)	84.7 (39.9) <sup><i>a</i></sup>
1/ $\sigma$	25.5 (2.3) <sup><i>a</i></sup>
<i>R</i> (I) <sub>merge</sub> (%)	6.3 (39.2) <sup><i>a</i></sup>
Molecular replacement model	Rab11a dimer
Refinement statistics	
Resolution range (Å)	10–1.25
Number of reflections	73,767
Number of atoms	Protein/GDP/water, 2662/56/508
R factors (%)	<i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub> , 13.9/19.6
r.m.s. deviation of bonds	Length/angle distance, 0.013 Å/0.032 Å

150 mM NaCl, 2.5 mM CaCl<sub>2</sub>) at 277 K, and precipitate was removed by centrifugation at 20,000 × *g* for 20 min at 277 K. To the soluble fraction, 1 unit of thrombin protease (Novagen) was added per milligram of fusion protein, and the His tag was removed by digestion for 4 h at 298 K (thrombin cleavage results in a Rab9a-(1–177) protein with an N-terminal GSHMAS extension). The thrombin cleavage reaction was diluted (1:3, v/v) with 20 mM MES, pH 6.5, and applied to Q-Sepharose (Amersham Biosciences), which had been previously equilibrated with Q buffer A (20 mM MES, pH 6.5, 50 mM NaCl). Native Rab9-(1–177) was eluted from Q-Sepharose with a 50–750 mM NaCl linear gradient in MES, pH 6.5; fractions containing native Rab9-(1–177) were identified by denaturing gel electrophoresis and pooled. The pooled Q fractions were then further purified by gel filtration on Sephacryl S-200 (Amersham Biosciences) in MES, pH 6.5, 150 mM NaCl; fractions containing Rab9-(1–177) were pooled and concentrated by ultrafiltration.

FIG. 3. **Ribbon representation of Rab9 structure.** A rainbow ramp color coding of *blue* to *red* is used to mimic chain trace from the N terminus to the C terminus. Both termini and the secondary structure elements are *labeled*. GDP in the active site is shown in *ball-and-stick* formation. *Gray*, carbon atoms; *blue*, nitrogen atoms; *red*, oxygen atoms; *purple*, phosphorus atoms.

sodium chloride with a protein concentration of 10 mg/ml. Crystals were grown at 277 K by the hanging-drop vapor diffusion method with 100 mM sodium acetate buffer, pH 5.0, 5% (v/v) polyethylene glycol 4000 as

FIG. 4. Stereoview of the active site showing the interactions between Rab9 and GDP. Gray, carbon atoms; blue, nitrogen atoms; red, oxygen atoms; purple, phosphorus atoms. The bonds in GDP are colored green.

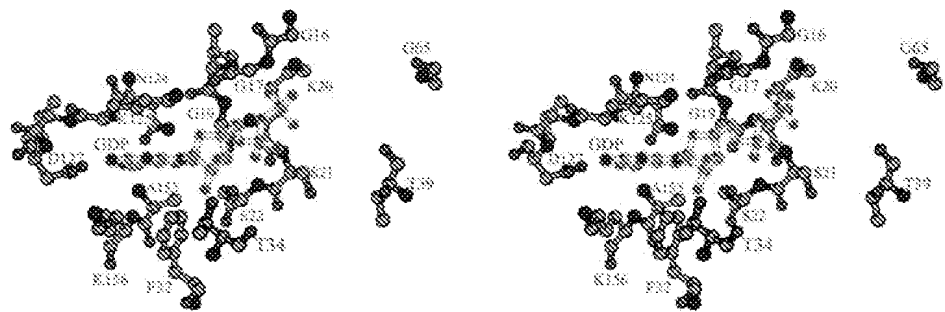


TABLE II  
Hydrogen bonds between Rab9 and GDP

Rab9 atoms	GDP atoms	Hydrogen bond distances
		Å
Monomer A		
Gly-17 N	O1B	2.83
Gly-19 N	O3A	3.13
Lys-20 N	O2B	2.85
Lys-20 NZ	O2B	2.81
Ser-21 N	O3B	2.91
Ser-22 N	O1A	2.84
Ser-22 OG	O1A	2.68
Thr-34 OG1	O3 <sup>a</sup>	3.02
Thr-34 OG1	O2 <sup>a</sup>	3.05
Lys-125 NZ	O4 <sup>a</sup>	3.04
Asp-127 OD1	N1	2.82
Asp-127 OD2	N2	2.83
Ala-155 N	O6	2.86
Monomer B		
Gly-17 N	O1B	2.89
Gly-19 N	O3A	3.13
Gly-19 N	O2B	3.02
Lys-20 N	O2B	2.88
Lys-20 NZ	O2B	2.75
Ser-21 N	O3B	2.96
Ser-22 N	O1A	2.93
Ser-22 OG	O1A	2.70
Thr-34 O	O2 <sup>a</sup>	2.50
Asn-124	N7	3.17
Lys-125 NZ	O4 <sup>a</sup>	2.99
Asp-127 OD1	N1	2.72
Asp-127 OD2	N2	2.83
Ala-155 N	O6	2.82

<sup>a</sup> Denotes atoms from sugar rings.

crystallization solution. Crystals formed in space group  $P_1$  with  $a = 38.40$  Å,  $b = 45.62$  Å,  $c = 51.22$  Å,  $\alpha = 99.8^\circ$ ,  $\beta = 107.2^\circ$ , and  $\gamma = 101.8^\circ$  and contained two monomers in the unit cell. X-ray diffraction data to 1.25-Å resolution were collected at beamline 22-ID in the facilities of the South East Regional Collaborative Access Team at the Advanced Photon Source, Argonne National Laboratory. The statistics for data collection and processing are summarized in Table I.

**Structure Determination and Refinement**—The orientation and position of the Rab9 dimer in the  $P_1$  unit cell were determined using the molecular replacement protocols in Crystallography & NMR System (14) starting from the structure of Rab11a (PDB code 1OIV (15)) as the search model. The composite omit map was calculated to guide electronic density fitting of the model. Energy-restrained crystallographic refinement was carried out with maximum likelihood algorithms implemented in Crystallography NMR software (14). Refinement proceeded through several cycles in combination with manual checking by the program O (16). The addition of two GDPs and 473 water molecules and refinement up to 1.25 Å resulted in  $R$  and  $R_{\text{free}}$  values of 0.213 and 0.232, respectively. Further refinement was continued with SHELX-97 (17) by subjecting the structure to cycles of isotropic conjugate gradient least squares refinement; then tightly restrained anisotropic displacement parameters were introduced and refined. The final refinement cycle resulted in  $R/R_{\text{free}}$  values of 0.139/0.196. The final model contains residues 2–34 and 39–175 of monomer A, residues 5–34, 39–110, and 115–175 of monomer B, and 2 GDP molecules plus 508 water molecules. The phasing and refinement statistics are summarized in Table I.

**Protein Fold Analysis**—Secondary structure elements were defined by the hydrogen-bonding patterns in combination with visual inspection. The

		I	II
P21Ras	1	MPEYKLVVVGAGGVNKSALTIQLQNNH	EYDFP-I-ENGY
Rab11a	6	EEEDYKLVVIGDSGVNKNLLSRFTNNEF	EEKK-IQVEFA
Ypt7p	1	MSSEKQGLKVIILGDSGVNKLHRRYVNDKY	SOQDKA-IGASFL
Rab9	1	MSGRSEELKVIILGDSGVNKLHRRYVTKNF	QLFH-IGVEFL
		B1	H1
		III	IV
P21Ras	41	RKQVVID-GETCLLDILDITA-QEETSAMRQYHRT	EGGFLOVFAIN
Rab11a	50	TRSIQND-GETIKAQIWDITA-QEETRAITTSAYY	RGAVGALLVYDIA
Ypt7p	47	TKEVTVDGKRVATQVWDITA-QEETGELGVAFIR	GCACCCVLVDVT
Rab9	46	NKIDLEVD-GRFTVMQIWDITA-QEETRSIRLREFF	IRGSUCCLLTPEVO
		B2	B3
		B2	B4
		V	VI
P21Ras	66	NTKSPEDIHQYREQIKRVRKDS---DQVPMVLNG	KNCLIA---RTVES
Rab11a	95	KHLTYENVERWLKELPDHADS---NIVIMLNGN	KSLIP-GERAVPT
Ypt7p	93	GASSPENIKSWHDEFIVSANVNSPTTEFFVIL	GNNICAEESKIVSE
Rab9	91	DGQSFPQLSNWKKFIYIYALVKEPESEPFVIL	GNNICIS---EPQVST
		H3	B5
			VII
P21Ras	128	RQAQDLAREYQ-IPYIETSAKTRQGVEDAFYTL	LVREIRQH
Rab11a	137	DEARAFAEKNG-LSPFETSALGSTNVSAFQTIL	ITRY
Ypt7p	140	KSAQELANSILGDIPLFLTSARKNINVDATAFER	IAREALQNG
Rab9	136	EEAQAWCPENGQDYFFETSAKGNATNVAAAFE	EAURVRLATEERSD
		R4	B6
			H5

FIG. 5. Structure-based sequence alignment of Rab9 with the most similar structures. The sequence number for the first residue of each line is indicated on the left. The positions of the secondary structural elements in Rab9 are indicated by underlining the sequence and labeling as in Fig. 1. Residues that are highly conserved in the Rab GTPase family are indicated in boldface type. Residues that are known to contact GDP in protein-GDP complex structures are indicated in red. Additional residues that would interact with the  $\gamma$ -phosphate in protein-GTP analog complex structures are shown in purple. The seven regions that show high degree of conformational variation among the superimposed structures (see Fig. 6) are highlighted in gold and labeled. See Table III for protein abbreviations and references.

Dali algorithm of comparing protein domain structures by alignment of distance matrices was used to search for structural homologues of Rab9 and also used for structure-based sequence alignment (18, 19). Ribbon diagrams were prepared by the program MOLSCRIPT (20).

## RESULTS

**Structure Determination**—The human Rab9 variant we used for crystal structure determination included residues 1–177, lacking its last 24 residues (Fig. 1). Known as the C-terminal hypervariable region, the amino acid sequence of this region in Rab9 is poorly conserved with respect to other Rab proteins. Therefore, we excluded the C-terminal 24 residues from our cloning and crystallographic studies. We will refer below to this truncated form of the protein as Rab9.

Rab9 bound to GDP was crystallized, and its structure was determined by molecular replacement (Table I). The structure was refined against 1.25-Å resolution data, making it one of the highest resolution structures in the Rab protein family. Both N and C termini (residues 1 and 176–177 of monomer A and residues 1–4 and 176–177 of monomer B) and some loop re-

## High Resolution Structure of Rab9 GTPase

TABLE III  
Alignment statistics of Rab9 with structurally similar proteins

Statistics were produced using the Dali algorithm (18, 19).

Protein name	Protein Data Bank code	Z <sup>a</sup>	RMSD <sup>b</sup>	LALI <sup>c</sup>	LSEQ2 <sup>d</sup>	IDE <sup>e</sup>	Ref.
GDP-bound Rab9	1WMS	35.3	0.0	168	168	100	This paper
Gpp(NH)p-bound Ypt7p	1KY2	26.6	2.0	167	180	53	22
Gppsp-bound Rab11a	1OIW	25.4	1.4	158	166	41	15
Gpp(NH)p-bound p21Ras	1CTQ	25.2	1.6	161	166	31	21
GDP-bound Rab11a	1OIV	25.1	1.6	161	168	43	15
GDP-bound Ypt7p	1KY3	24.9	1.5	153	162	54	22
Gpp(NH)p-bound Sec4	1G17	24.7	1.6	159	168	38	7
Gpp(NH)p-bound Rab5a	1N6H	24.2	1.8	160	167	38	23
Gpp(NH)p-bound Rab5c	1HUQ	24.0	1.8	159	164	39	24
GDP-bound Sec4	1G16	23.3	1.5	150	156	35	7
Gpp(NH)p-bound Ypt51	1EK0	23.2	1.8	159	168	40	6

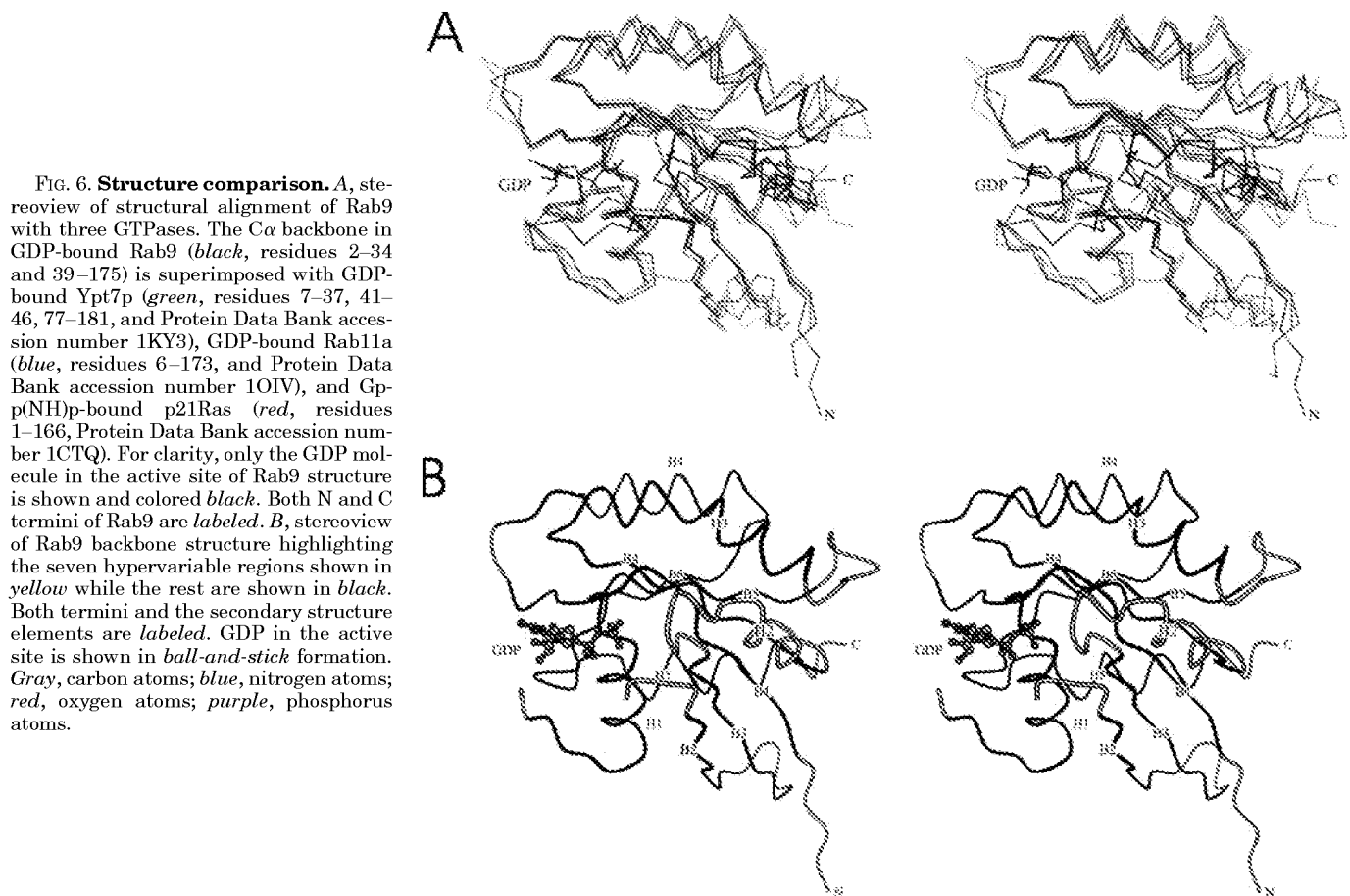
<sup>a</sup> Z-score, strength of structural similarity in standard deviations above expected.

<sup>b</sup> Positional root mean square deviation of superimposed C $\alpha$  atoms in Å.

<sup>c</sup> Total number of equivalent residues.

<sup>d</sup> Length of the entire chain of the equivalent structure.

<sup>e</sup> Percentage of sequence identity over equivalent positions.



regions (residues 34–38 of both monomers and residues 111–114 of monomer B) were disordered and could not be seen in the experimental electron density map. The final refined model, which includes residues 2–34 and 39–175 of monomer A, residues 5–34, 39–110, and 115–175 of monomer B, 2 GDP molecules, and 508 ordered water molecules, has a working *R* value of 0.139 and a free *R* value of 0.196. The stereochemistry is excellent with r.m.s. deviations for bond lengths and angle distances of 0.013 Å and 0.032 Å, respectively (Table I). The Ramachandran plot statistics showed that 93.2% of the backbone dihedral angles were in the most favored regions, 6.8% in the additional allowed regions, and none of the non-glycine

residues were in the disallowed regions. The two crystallographically unique Rab9 molecules in the crystal unit cell have almost identical structures with the r.m.s. deviation between the 161 equivalent C $\alpha$  atoms of 0.40 Å. We will use monomer A in our description of Rab9 structure.

**Overall Structure of Rab9**—Like other members of the Rab GTPase family, Rab9 adopts a classical nucleotide binding fold consisting of a six-stranded  $\beta$ -sheet surrounded by five  $\alpha$ -helices (Figs. 2 and 3). The five  $\alpha$ -helices (H1–H5) and six  $\beta$ -strands (B1–B6) connect with a B1–H1–B2–B3–H2–B4–H3–B5–H4–B6–H5 topology containing 30.5% (54/177)  $\alpha$ -helix, 28.8% (51/177)  $\beta$ -sheet, 37.3% (66/177) turn/loop, and 3.4% (6/177) other (Figs.

1 and 3). The six  $\beta$ -strands arrange in the order of B2-B3-B1-B4-B5-B6, forming a mostly parallel  $\beta$ -sheet except that B2 is antiparallel to the rest in strand direction.

**Active Site Structure**—The crystal structure reported here contains a tightly bound GDP molecule in the active site (Figs. 3 and 4). Rab9 residues making hydrogen bonds with GDP include Gly-17, Gly-19, Lys-20, Ser-21, Ser-22, Thr-34, Asn-124, Lys-125, Asp-127, and Ala-155 (Table II, Fig. 4, and as shown by the color red in Fig. 5). These residues together with Gly-16, Val-18, Phe-32, Ile-126, and Lys-156 form a tight binding pocket accommodating the GDP molecule. There are more than a dozen hydrogen bonds formed between Rab9 and GDP, indicating a strong binding affinity of Rab9 for GDP. This is supported by the observation that the GDP molecule has been kept inside the active site throughout Rab9 purification, that is it comes naturally bound to Rab9 from the cell culture. Residues Thr-39 and Gly-65 are highly conserved in the Rab GTPase family and are predicted to interact with the  $\gamma$ -phosphate of GTP in the active state of Rab9.

#### DISCUSSION

**Structure Comparison**—The overall structure of Rab9 is very similar to the prototype Ras protein p21Ras (21) and several Rab proteins (Table III). Among those, Rab9 has the highest sequence identity with Ypt7p (54% over 153 equivalent positions), followed by Rab11a (43% over 161 equivalent positions). The structural similarity Z-scores (18, 19) range from 26.6 to 23.2 with r.m.s. deviations of equivalent positions in the range of 1.4–2.0 Å. Structure-based sequence alignment reveals that the active site of Rab9 consists of residues highly conserved in the Rab GTPase family (Fig. 5), implying a common catalytic mechanism. However, Rab9 contains seven hypervariable regions that are significantly different in conformation from other Rab proteins (Figs. 5 and 6). Some of those regions coincide with putative effector-binding sites and conformational switch I and switch II regions identified by earlier crystallographic studies of other Rab proteins. Regions II and IV correspond to the switch I and switch II, respectively, whereas regions I, V, and VII correspond to the three effector-binding sites/complementary determining regions. These seven hyper-

variable regions in Rab9 structure may serve as sites for antiviral drug binding and provide an excellent target for structure-based drug design and development.

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